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DEVELOPMENT OF IN VITRO ISOLATED PERFUSED PORCINE SKIN FLAPS FOR STUDY OF PERCUTANEOUS ABSORPTION OF XENOBIOTICS

ANNUAL REPORT

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This report describes the development of a novel in vitro alternative animal model for dermatology and cutaneous toxicology. An anatomically intact, viable, isolated perfused skin preparation would be a useful model for studying percutaneous drug because venous and arterial perfusate concentrations could be assessed independently of confounding systemic processes. In order to develop such a model, a single-pedicle, axial-pattern, island-tubed

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FIGURE 2

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skin flap was created in crossbred Yorkshire weanling pigs in one surgical procedure, then transferred 2 or 6 days later to a computer-controlled, temperature-regulated perfusion chamber for 10-12 hour studies. The development of this two stage surgical procedure is fully described. Pig skin was used because of its recognized similarity to human skin. Perfusate consisted of Krebs-Ringer bicarbonate buffer (pH=7.4) containing albumin and glucose. Viability was assessed by glucose utilization, lactate production, and an absence of significant concentrations of the intracellular enzyme lactate dehydrogenase in the perfusace. Light and electron microscopy was used to develop a morphological viability index and to differentiate degenerate lesions from normal surgery or perfusion changes or lesions from exogenously applied toxins (e.g., sodium fluoride (NaF). Based on these criteria, biochemically viable skin flaps could be maintained for 12 hours without significantly abnormal morphology. A mean lactate to glucose ratio of 1.7 suggested primarily anerobic glycolysis. The research conducted during this period has resulted in a reproducible perfusion model optimized for the xenobiotic absorption studies to be conducted in the second year. This preparation would be an humane alternative animal model for studies in cutaneous toxicology, physiology, oncology, and percutaneous drug absorption and metabolism.

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This report describes the development of a novel in vitro alternative animal model for dermatology and cutaneous toxicology. An anatomically intact, viable, isolated perfused skin preparation would be a useful model for studying percutaneous drug absorption because venous and arterial perfusate concentrations could be assessed independently of confounding systemic processes. In order to develop such a model, a single-pedicle, axial-pattern, island-tubed skin flap was created in crossbred Yorkshire weanling pigs in one surgical procedure, then transferred 2 or 6 days later to a computer-controlled, temperature-regulated perfusion chamber for 10-12 hour studies. The development of this two stage surgical procedure is fully described. Pig skin was used because of its recognized similarity to human skin. Perfusate consisted of Krebs-Ringer bicarbonate buffer (pH=7.4) containing albumin and glucose. Viability was assessed by glucose utilization, lactate production, and an absence of significant concentrations of the intracellular enzyme lactate dehydrogenase in the perfusate. Light and electron microscopy was used to develop a morphological viability index and to differentiate degenerative lesions from normal surgery or perfusion changes or lesions from exogenously applied toxins (e.g., sodium fluoride <NaF>). Based on these criteria, biochemically viable skin flaps could be maintained for 12 hrs without significantly abnormal morphology. A mean lactate to glucose ratio of 1.7 suggested primarily anaerobic glycolysis. The research conducted during this period has resulted in a reproducible perfusion model optimized for the xenobiotic absorption studies to be conducted in the second year. This preparation would be an humane alternative animal model for studies in cutaneous toxicology, physiology, oncology, and percutaneous drug absorption and metabolism.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Studies on the function of skin have been limited by a lack of in vitro models for investigating cutaneous physiology in an anatomically and physiologically intact preparation. This limitation is especially evident when those studies require measuring arterial-venous concentration differences of endogenous compounds metabolized or produced by the skin. Current techniques assessing the percutaneous absorption of drugs and chemicals are severely limited if the absorbed compounds are extensively metabolized by the skin or form cutaneous depots (1). An anatomically intact, viable, isolated, perfused tubed skin preparation would overcome many of these limitations because perfusate composition could be controlled rigidly, allowing comparison of the arterial and venous compositions of perfusate during assessment of cutaneous metabolism and function. Pigs were selected because their skin is functionally and structurally similar to that of man (2,3,4,5,6,7,8). Earlier studies utilized flat skin flaps in dogs (9,10), which as described, were not amenable to long-term computer-controlled experiments with skin exposed in an ambient environment. Skin in the caudolateral epigastric region of the pig has direct cutaneous vasculature, the caudal superficial epigastric artery (CSEa) and its paired venae comitantes, which is compatible with closed isolated organ perfusion techniques.

Overview of Surgical Procedure:

The arterial blood supply to the dermal-subdermal plexi of skin will traverse three types of vasculature: 1) the segmental, 2) the perforators, and 3) the cutaneous. In general, the venous return parallels the arterial supply. Segmental vessels arise from the aorta, lay deep to the muscle mass, and usually follow the course of a peripheral nerve. Perforating vasculature functions by supplying blood to the muscles through which it passes and serves as a conduit from the segmented vasculature to the cutaneous circulation. The cutaneous arterial supply is subdivided into two types: 1) the musculocutaneous arteries and 2) the direct cutaneous arteries. In man and pig, the main blood supply to the skin is via many musculocutaneous arteries which penetrate directly from muscle through the subcutaneous tissues and into the overlying skin. Musculocutaneous arteries supply relatively small areas of skin. The musculocutaneous arterial system is supplemented by limited numbers of anatomically variable direct cutaneous arteries which course parallel, instead of perpendicular, to the skin at a level above the muscle and fascia. Direct cutaneous arteries supply much greater areas of skin. Unlike man and pig, the dog and other loose-skinned animals do not have a musculocutaneous arterial system; their primary vascular supply to the skin is from direct cutaneous arteries.

A skin flap consists of skin and subcutaneous tissue that is moved from one part of the body to another with a vascular

pedicle or attachment to the body being maintained for nourishment. Skin flaps are classified based upon their blood supply, or by the location to which they are moved (i.e., local or distant). Pedicles of random pattern flaps are usually supplied by musculocutaneous arteries, which in turn perfuse the dermal-subdermal plexi of the skin flap. The surviving length of random-pattern flaps is related to the arterial perfusion pressure and venous drainage; however, these skin flaps can be made larger (50 to 100 %) if they are "delayed." When a skin flap is designed to include a direct cutaneous artery within its longitudinal axis, it is called an axial-pattern flap. Axial-pattern flaps are further subclassified as 1) peninsular flaps, having direct cutaneous vasculature with an intact skin/subcutaneous tissue bridge; 2) island flaps, lacking a skin/subcutaneous tissue bridge, but attached to a vascular supply; and 3) free flaps, which are transplanted to distant sites and connected to recipient vasculature by microvascular anastomosis.

The surviving length of an axial-pattern flap is determined by the extent of the direct cutaneous artery in the flap plus that distal skin which is perfused through its dermal-subdermal plexus. Island flaps survive to at least the same length as peninsular flaps when both are made under similar conditions. Axial-pattern flaps often survive to 50% greater length than "delayed" random flaps, and axial-pattern flaps can be made longer by delay procedures.

Among the several types of distant skin flaps is the tubed flap. A tube flap is bipedicled (i.e., attached at both ends), which has been raised in an area of abundant skin and its parallel edges sewn together to form a tube resembling a suitcase handle. The skin beneath the tubed flap (i.e., the donor site) is either undermined and closed by primary suture or grafted. Thereafter, the tubed flap can be detached one end at a time and "tumbled," "waltzed," or "caterpillared" to the distant, recipient site. After a tubed flap is raised, vascular transformation occurs; however, the change in random-pattern tubed flaps and axial-pattern tubed flaps is different.

The objectives of the surgical phase of this study were 1) to identify and establish the normal vasculature to proposed skin flap donor sites and 2) to develop a single-pedicle, axial-pattern tubed flap which can 1) be raised in one operation, 2) survive to its entire length, 3) be characterized regarding its physiological characteristics during healing, and 4) be harvested for transfer to the <u>in vitro</u> percutaneous absorption studies.

Morphological Viability Studies:

The objective of this phase of the study was to assess and quantitate over time the changes that occur in skin 1) as a result of normal cell death, 2) as a function of the surgical procedure used in creating the flap, 3) as a result of isolated perfusion, and 4) to determine changes which occur secondarily to

chemically-induced toxicity. A classification of these structural changes is essential for the proper application of morphologic criteria as a discriminator between cell death, surgery, perfusion and chemically-induced toxicity in the isolated perfused porcine skin flap (IPPSF).

This report describes the development of a novel isolated perfused organ system, the IPPSF. The criteria used to establish viability are described, as well as preliminary studies of the functional and anatomic integrity of this preparation. The successful perfusion of 29 IPPSF's (a representative 18 of which are described in detail with the other 11 IPPSF's not being perfused to the final standardized protocol), in which viability was maintained for periods of 10 hours or longer, is also described.

MATERIALS AND METHODS

Development of Surgical Procedure:

Experiment 1: Two weanling female pigs weighing approximately 40 kg were anesthetized, exsanguinated and embalmed with injectable latex. Prosection of the pelt was done to elucidate the pattern of cutaneous vasculature in three prospective skin flap donor sites (i.e., buttock, lateral thoracic and caudal abdominal regions) (11,12,13,14,15) and to provide reference anatomical preparations.

Experiment 2: Two female pigs were anesthetized. Stainless steel staples were applied to the skin bilaterally, defining the limits (4 x 12 cm) of the proposed skin flap donor site. Incision and blunt dissection exposed the femoral and CSE arteries. The arteries, in order, were cannulated to allow infusion of 60% meglumine iothalamate for <u>in vivo</u> angiography of the cutaneous vasculature. Following completion of these studies, the pig was heparinized and exsanguinated, its thoracic aorta was cannulated, and 2-3 L of micropulverized barium sulfate-gelatin solution was infused under controlled constant pressure. The pig was skinned after the gelatin had solidified and the abdominal pelt was contact-radiographed to determine the architecture of the normal cutaneous vasculature.

Experiment 3: Six female pigs were anesthetized and prepared for aseptic surgery. Random (CSEa-ligated, n=2) and axial-pattern (n=10) skin flaps (2 flaps/pig) of dimensions based on the above studies, those of others, and recommendations for design of tubed flaps were raised, based on the CSEa. Fluorescein was infused and the line demarcating perfused/nonperfused portions ("surviving length") of the skin flaps, if any, was marked with stainless steel skin staples. The pigs were recovered from anesthesia. Two to 7 days later, each pig was anesthetized and heparinized. Fluorescein angiography was repeated and the surviving length of each skin flap was recorded. Thereafter, the pig was exsanguinated and either the

aorta (random-pattern skin flaps) or CSEa (axial-pattern skin flaps) was cannulated to allow barium sulfate-gelatin infusion under controlled constant pressure. The skin flaps were resected, pinned to original size and contact-radiographed to allow comparison with fluorescein angiography and surviving length data.

Experiment 4: This experiment comprised two-stage surgical procedure for routine preparation (stage 1 procedure) of single-pedicle, axial-pattern tubed flaps (Figure One). In the stage 1 procedure, each female pig was premedicated with atropine sulfate (.04 mg/kg i.m.) and xylazine hydrochloride (0.2 mg/kg i.m.) and maintained with halothane delivered by endotracheal tube. Each female pig was prepared for routine aseptic surgery in the caudal abdominal and inguinal regions. The proposed skin incisions and reference marks for wound margin alignment and skin flap retraction/expansion studies were outlined in the caudolateral epigastric region using a sterile marking pen. The skin incisions were made in order (medial, cranial and lateral [peninsular stage 1 procedure, n=6] and caudal [island stage 1 procedure, n=6]) with a No. 10 scalpel blade and extended to the level of the muscular fascia. Craniomedial branches of the CSEa that supplied the caudal mammae were ligated and divided. Using skin hooks, the wound margins of the medial incision were retracted for direct visualization of the CSEa, allowing scalpel dissection of the subcutaneous tissue and skin flap elevation without vascular damage. In island stage 1 tube flaps, the caudal incision was realigned and sutured with a size 3-0 polypropylene suture, using modified 3-point sutures to anchor the corners and a simple continuous pattern for the remaining wound. The tubed flap was formed and trimmed minimally of fat at its edges, if necessary. The tubed flap skin edges were closed using size 3-0 polypropylene in a simple continuous pattern. Starting 2.5 cm cranial to the base of the tubed flap, the subcutaneous tissues were apposed with five to seven vertically oriented, interrupted retention sutures using size 2-0 chromic gut. The superficial subcutaneous tissue was closed with 2-0 chromic gut in a simple continuous pattern. The skin incisions at the donor site and base of the flap were closed using size 2-0 nylon in simple continuous and simple interrupted patterns, respectively.

Fluorescein angiograms were evaluated 12 minutes after infusion of 5 ml of 10% fluorescein to predict surviving length of all tubed flaps. Thereafter, the surgical site and tubed flap were bandaged using a self-adherent wound dressing[c] that had been affixed to the skin using size 3-0 nylon in a continuous cruciate pattern. The pigs were recovered from anesthesia and housed individually.

The healing of axial-pattern tubed flaps was evaluated by visual inspection, fluorescein angiography and determination of tissue shrinkage during the postoperative period. This experiment was considered to be successful when it was determined that

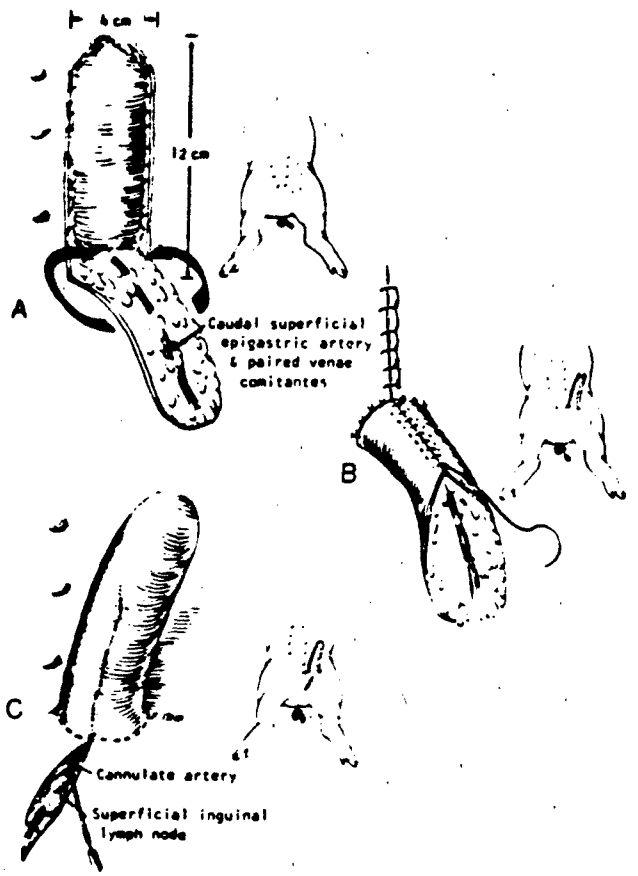

Fig. 1. Surgical procedures for preparation of <u>in vitro</u> isolated perfused porcine skin flaps.

a. Stage I Procedure: Single-pedicle, axial-pattern tubed flap, supplied by the caudal superficial epigastric artery and its paired venae comitantes, is raised on weanling female Yorkshire pigs.

b. The donor site and tubed flap are closed primarily, secured to the surrounding abdominal skin via one or two sutures, and bandaged.

c. Stage II Procedure: Two to 6 days later, the tubed flap is harvested following cannulation of the caudal superficial artery and one of the venae comitantes (when possible), and transferred to the isolated organ perfusion apparatus.

a single-pedicle, axial-pattern tubed flap could be raised and survive the entire length for an appropriate postoperative period.

In the stage 2 procedure, each female pig was preanesthetized, induced and maintained on anesthesia as described above. Visual inspection or fluorescein angiography was performed as described above to ascertain that the skin flap in each island stage 1 procedure had survived to its entire length. Each pig was prepared for routine aseptic surgery in the caudal abdominal and inguinal regions. A skin incision measuring 6 cm was made in the inguinal region which extends caudally from the base of the tubed flap (Figure One c). Using blunt dissection, the incision was deepened to the level of the superficial inguinal lymph node, thereby exposing the CSEa, which emerged from its deep surface. At this time, the pig was heparinized (3,000 iu i.v.) and the exposed vasculature bathed with 1-2 ml of 2% lidocaine hydrochloride to minimize vasospasm during subsequent manipulations. Aided by 3X microsurgical lenses, the CSEa was isolated between two stay sutures of size 3-0 polypropylene. An opening in the wall of the CSEa was established and extended, using delicate 45 Potts cardiovascular scissors. The CSEa was cannulated with PE20 tubing, which was secured by the stay sutures. The patient side of the pedicle containing the caudal superficial epigastric vasculature was cross-clamped and the tubed flap resected. Heparinized, balanced polyionic saline solution (approximately 20 ml) was infused via the arterial cannula to clear the tubed flap of blood and establish that patency of the venae comitantes existed. The tubed flap was transferred to an assistant for transport in a clean tray to the isolated organ perfusion laboratory. The surgeon returned to the operative site on the pig and the vascular pedicle was double-ligated with size 0 chromic gut. The wound was flushed and inspected to ensure adequate hemostasis and ventral drainage. The pig was recovered from anesthesia and the wound allowed to heal by second intention.

Experiment 5: Nine groups of weanling female Yorkshire pigs (n=25) were anesthetized and the newly developed single-pedicle, axial-pattern tubed flap was raised and allowed to heal for 0 days (group 2, n=4), 2 days (group 3, n=7), 6 days (group 4, n=6), 14 days (group 5, n=2; group 6, n=1), and 28 days (group 7, n=1; group 8, n=1). In groups 2, 3, 4, 5 and 7, the axial-pattern flap was designed in the island configuration; in groups 6 and 8, the peninsular configuration was used. Normal skin for comparison was obtained from the caudolateral abdominal region (group 1, n=2). Eight of 25 tubed flaps were perfused <u>in vitro</u> successfully prior to preparation for microangiographic and histologic examination. Each female pig was anesthetized at the conclusion of its healing period and fluorescein angiography was repeated to assess the surviving length (100% was expected). The tubed flap was resected as described for the stage 2 procedure; its axial vasculature was cannulated and prepared for microangiographic examination as described above. The donor site was closed primarily and the pig was recovered from anesthesia.

Serial section microangiography and correlative histology of the harvested axial pattern tubed flap was done to establish the vascular pattern and skin morphology during healing. Microangiographic techniques were adapted successfully from established methods for examination of kidneys (16). Medial-lateral and dorsal-ventral views of each tubed flap were obtained to assess technical success and provide information regarding overall vascularity. Each tubed flap was sliced into 0.5 cm sections and four of these sections, one each from the base and tip regions and two from the midbody, were selected randomly. Microangiograms of 500 um sections of the selected slices were prepared with the remaining portion of each slice being prepared and stained with hematoxylin and eosin (H&E) for histologic evaluation.

This experiment was considered to be completed successfully when the results of vascular transformation in the tubed flap, in conjunction with other findings (detailed below), allowed determination of the appropriate postoperative time for tubed flap harvest, which will allow satisfactory percutaneous absorption studies.

Isolated Perfusion Protocol:

Single-pedicle, axial-pattern, tubed flaps supplied by the CSEa were raised on female weanling Yorkshire pigs, weighing approximately 20 kg (stage I surgical procedure). Based on the experiments above, landmarks for a caudolateral epigastric flap measuring 4 cm x 12 cm were utilized. Venous drainage was provided by the paired venae comitantes associated with the artery. Two or 6 days later, the tubed flap was harvested (stage II surgical procedure) and transferred to the perfusion apparatus following cannulation of the artery. After the tube flap was harvested and the donor site healed completely, the pig was returned to the animal housing facility for later resale.

Perfusion media consisted of a Krebs-Ringer bicarbonate buffer solution (pH=7.4) containing the following in g/L(mM): NaCl, 6.89(117.9); KCl, 0.36(4.8); $CaCl_2$, 0.28(2.5); KH_2PO_4, 0.16(1.2); $MgSO_4 \cdot 7H_2O$, 0.30(1.2); $NaHCO_3$, 2.75(32.7); glucose, 0.90(5.0); bovine serum albumin (Cohn fraction V), 45(0.7); gentamicin sulfate, 0.02(0.04); amphotericin B, 0.001(0.001); penicillin G, 10,000 IU; and sodium heparin, 5,000 USP. The perfusion apparatus (Figure Two) was a closed, recirculating system regulated for the relatively low perfusate flow rates (0.3 to 5.5 ml/min) seen in skin flaps. The apparatus was enclosed in a humidified plexiglass chamber maintained at 37°C. Medium was gassed with a mixture of 95% oxygen and 5% carbon dioxide using a silastic-tube oxygenator (17). A variable-rate peristaltic pump circulated 250 ml of medium to the cannulated artery of the skin flap. The perfusate was recirculated at a higher flow rate in an arterial-venous shunt line to provide adequate mixing of the medium. Glucose and sodium bicarbonate were infused into this

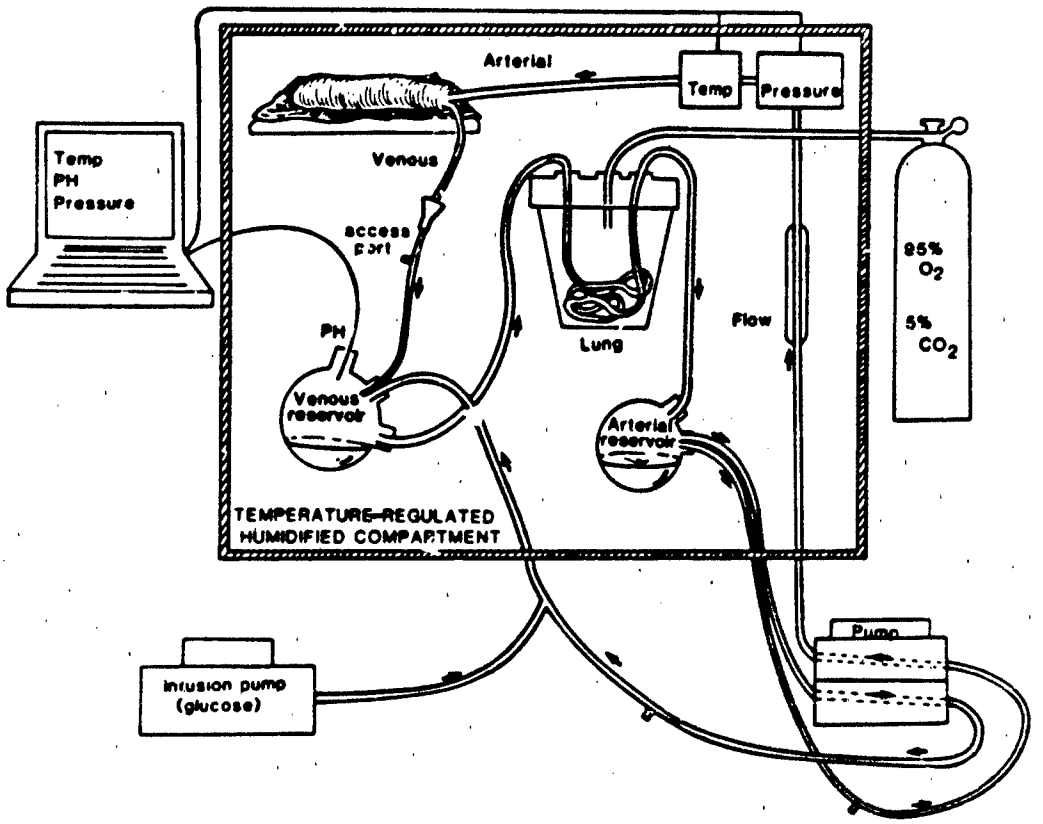

Fig. 2. Schematic of the isolated perfused skin flap apparatus.

shunt line to maintain arterial glucose concentrations between 80 and 120 mg/dl and stable perfusate pH (7.4).

Arterial perfusate pressure and temperature were constantly displayed and stored on a microcomputer. Arterial (2.5 ml) and venous (0.7 ml) perfusate samples were collected hourly without volume replacement for the determination of glucose concentrations (mg/dl), using an automated assay system (Glucose Analyzer 2; Beckman, Brea, CA), and osmolality (mOsm/kg; Osmette A; Precision Systems, Sudbury, MA). Glucose utilization (mg/hr) was calculated from the product of glucose extraction (mg/dl) and the flow rate (ml/min) at each observation time. Cumulative glucose consumption was estimated from the area under the glucose clearance versus time curve using the trapezoidal rule. Arterial pH, lactate and lactate dehydrogenase activity (LD) were also monitored to biochemically assess the flaps' viability. Lactate was assayed using Sigma Method 826-UV (Sigma Chemical Co., St. Louis, MO). LD, an intracellular enzyme, was used as a marker for cell membrane integrity (18) and was determined using a multistat centrifugal analyzer (MCA III^{+}; Instrumentation Laboratories, Lexington, MA).

Initial experiments (n=3) were not terminated at a specific time in order to determine markers of flap death. Subsequent flaps were terminated at 10-12 hours. Sodium fluoride (NaF), an inhibitor of glycolysis (19), was administered after 5 or 11 hours of perfusion (n=4) in order to assess the flap's sensitivity to metabolic inhibition. At the termination of each IPPSF experiment, skin samples were collected and processed for both light (LM) and transmission electron (TEM) microscopy.

Morphological Viability Assessments by Light and Transmission Electron Microscopy:

If a morphologic viability index was to be developed for the IPPSF, viability must first be defined. Viability in an IPPSF has a different connotation from that employed in cell culture work. In the flap, the majority of cells must be metabolically active, while in cell culture, only a small percentage of cells must be capable of subsequent growth on culture medium and cells may be in various dormant phases. Therefore, our criteria for viability, both morphological and biochemical, were more stringent than others. Morphologic changes due to cell death, surgery, toxicity, isolated perfusion or chemically-induced toxicity must be differentiated before a meaningful index could be constructed.

In order to define changes due to cell death, a 4 cm x 12 cm piece of skin was harvested from the normal site. Samples of skin were pinned to dental wax in a dissecting tray and floated on a 37^{o}C water bath. The samples were kept moist only on the dermal side by bathing with non-oxygenated lactated Ringer's in which antimicrobials were added (10 ug/ml gentamicin and 0.2 ug/ml amphotericin B). Six millimeter biopsy samples were taken from different locations on the flap based on a random digit table at

0, 15, 30, and 45 minutes and 1, 2, 3, 4, 8, 12, 24, 48, and 72 hours in three pigs. In two additional pigs, samples were taken at 1, 2, 4, 5, 6, 8, 9, 10, 12, 18 and 24 hours, The 6 mm biopsy was further divided in half for LM and the other half was divided between TEM and frozen samples. LM sections for each time period were independently scored by two investigators. Quantitative measurements were first based on basal dark nuclei, pyknotic basal cells, basal vacuoles, stratum spinosum dark nuclei, pyknotic stratum spinosum, and stratum spinosum on a scale from 1=(0-5 cells), 2=(5-10), 3=(10-20), 4=(20-40), 5=(diffuse). The second assessment was a subjective rating on the overall appearance of a section, scored as 1 (no change-normal), 2 (mild changes) and 3 (obviously necrotic). This rating was then correlated to the individual assessment. In order to improve the discriminatory ability of the information collected, the total number of epidermal cells were counted for each slide. The percent of each cell type observed in the above evaluation was calculated using the midpoint of each range as the cell count. Three alternative scales were also evaluated: percentage (%), square roots (% + 0.5) and logarithm (log(%+1)). Each of the latter scales resulted in the misclassification of three to four more slides than the original scales and were not considered further.

In order to define changes that occur in in situ flaps post-surgery, a biopsy study was performed in situ for 7 days after creating the flap in the stage I procedure. Morphometric analyses of LM samples were performed on three in situ skin flaps to help determine the optimal time of harvest following stage I surgery. Sections were collected daily on the flaps, from 0 (30 minutes after stage I surgery) to 7 days, at random sites along the length of the skin flap whose free end was sutured to the body wall to prevent traumatic injury. Pigs were immobilized with 0.2 cc of Rompun and 3 cc of ketamine. During these sampling procedures, the pig felt no pain, since cutaneous innervation was severed during the formation of the tubed flap. Paraffin-embedded sections, stained with H&E, were examined with a 40X objective attached to an Olympus PM-10ADS (Olympus Corp., Washington, D. C.) automatic photomicrographic system, using a calibrated eyepiece reticle. Each section was evaluated at four points, two each at thin epidermal areas and two at the thickest areas, coinciding with rete pegs. Thickness of the stratum corneum was constant at all times and was not analyzed. Data in the tables and figures are reported as mean $\pm$ SE.

In order to define changes specifically related to isolated perfusion, IPPSF tissue samples were taken after 12 hours of perfusion. Finally, in order to determine whether the IPPSF was responsive to chemically-induced toxicity, 10 mg/ml of NaF was administered to four flaps after 5 and 12 hours of stable perfusion. Glucose extraction ceased 40-90 minutes later.

Tissue processing techniques for LM, TEM and histochemistry are routinely used in our laboratory. Specimens for LM are fixed

overnight in half-strength Karnovsky fixative at 4°C (2% paraformaldehyde and 2.5% glutaraldehyde <976 mOSM> in 0.1 M cacodylate buffer), routinely processed, embedded in paraffin and stained with H&E or periodic acid Schiff (PAS). For enzyme histochemistry, small tissue pieces are quenched in an isopentane well in a Dewar flask filled with liquid nitrogen and stored at -70°C until studied. Tissue is then mounted on a stub with OCT compound and sectioned on an AO cryostat. Histochemical techniques presently being utilized in our laboratory on frozen sections include lactate dehydrogenase (20), acid phosphatase (21,22) ATPase (23) and nonspecific esterase (24). These should be useful for characterizing cell death and toxicity in IPPSF studies.

For TEM, tissue samples (1 mm^3) were fixed overnight in half-strength Karnovsky's (4°C, pH 7.4, 976 mOSM), postfixed in 1% osmium tetroxide for 1 hour, dehydrated through graded ethanol solutions and infiltrated and embedded in Spurr's resin, placed in flat embedding molds and polymerized in a 70°C oven overnight. Thick sections approximately 1 u were stained with toluidine blue for orientation. Ultrathin sections approximately 600 A° were sectioned with a diamond knife on a Reichert Ultracut E microtome (AO Reichert, Buffalo, NY). Sections were picked up on 300 mesh copper grids and stained with uranyl acetate and lead citrate and sections were then examined on a Philips 410LS transmission electron microscope (Phillips, Mahwey, NJ)at an accelerating voltage of 60 or 80 kv.

RESULTS

Development of Surgical Procedure

Experiment 1: Prosection of the "omnipotential pig buttock flap" as a proximally based island arterial flap (sic) (14,15). based on the deep circumflex iliac artery (DCIa) was done and it was found that the caudal 8-10 cm of this flap was vascularized by musculocutaneous perforators. Although the DCIa provided a direct cutaneous arterial system to the cranial portions of that flap, it was judged difficult to isolate and cannulate surgically without dissection of the medial thigh region of the pig. During dissection, it was found that muscle adhered to the skin in those regions in which the cutaneous vascular supply was musculocutaneous in nature. Because the skin flap was thick, it would be hard to tube, and primary closure of the donor site would not be possible. Therefore, further development of the "omnipotential pig buttock flap" as a donor skin flap was abandoned.

Inspection of the ventrally-based "trunk flaps" (sic) (11,12) indicated that development of an axial-pattern tubed flap based on this donor site should be discontinued for lack of a well-developed direct cutaneous arterial system and the presence of thick cutaneous trunci (paniculus carnosus) muscles.

In the caudolateral abdominal region, it was found that both the left and right CSEa's followed similar distribution patterns: 1) origin, 2) caudomedial branches and 3) cranial extension, which divides into a lateral portion supplying skin lateral to the line of mammae and craniomedial branches supplying each of the caudal mammae and median skin. Two venous systems were observed in the caudal abdominal skin region: 1) paired venae comitantes in association with the CSEa and 2) a cranial superficial epigastric venous system with an anastomotic branch to the deep circumflex iliac vein and an ill-defined caudal extent that terminates in the region of the caudal mammae. Approximately 14-16 cm cranial to the caudalmost teat, i.e., at the level of the caudal extent of the cutaneous trunci muscle, the abdominal skin becomes nourished by musculocutaneous vasculature.

Based on the assessment of these results, an axial pattern tubed flap (10 x 4 cm) incorporating the skin perfused by the CSEa was considered feasible.

Experiment 2: Both angiography and contact radiography of the abdominal pelt confirmed the observations in experiment 1. Nonselective aortic angiography readily identified the pudendoepigastric arterial trunk and its subsequent distribution, including the CSEa, to the flank and abdominal skin. Using either nonselective aortic or selective CSEa angiography, CSEa branches coursed the entire length of the proposed skin flap. Communication between the two venous systems were observed; subsequent experience has indicated that enlargement of the venae comitantes is obvious between days 2 and 29 in response to separation of the raised skin flap from the large venous system associated with the mammae.

Experiment 3: All caudolateral epigastric skin flaps survived to a level at least 13 cm cranial to the caudalmost teat (Table 1); however, filling of an axial vessel was seen in peninsular or island flaps only. Although additional replicates would be needed to correct the data for variability between pigs, e.g., weight and skin flap retraction, and to establish standard deviations for all measured parameters, certain trends have become apparent. The surviving lengths of island flaps $\geq$ peninsular flaps > random flaps. One could expect approximately 10-15% greater surviving length of any flap 2-7 days later, compared to predicted survival at time 0, using our method of fluorescein angiography. Fluorescein angiography at 2-7 days postoperatively correlated consistently with surviving length of the skin flap. Vasculature was observed in the surviving portions of all skin flaps, but did not correlate as closely with surviving length as did fluorescein angiography. The microangiographic studies in this experiment did not allow us to elucidate clearly the venous drainage from the base of island versus peninsular skin flaps. Based on these studies, it was concluded that a single-pedicle, axial-pattern (either island or

Table 1: Comparison of fluorescein angiography, surviving lengths, and microangiography data in various abdominal skin flaps.

Flap Type	Size*	Initial Retraction (in length)	Fluorescein Angiography† 0	48h	72h	7d	Later Retraction (in length)	Surviving Length† 48h	72h	7d	Vessels Seen in Radiographs†
Peninsular Flap ("ventral type I," n=1)	12x12	...	17.5	21.7	...	...	-12%	21.7	...	...	19.0
Peninsular (n=4)	6x12	-6%	17.0	23.0‡	17.7‡	19.0‡	-14%	23.0‡	17.7‡	19.0‡	17.6
Island (n=4)	6x12	-6%	18.7	...	19.7‡	22.5‡	-12%	...	19.7‡	22.5‡	20.5
Random (n=2)	6x12	-6%	8.8	...	...	13.2	-17%	...	...	13.2	12.8
Island (denervated, n=1)	6x12	...	19.7	...	...	21.2	-16%	...	...	20.2	16.0

*Dimensions in cm.
†Distance in cm (±) from base of flap.
‡n=1
§n=2
‖n=3

peninsular), tubed flap measuring 4 x 12 cm would probably survive to its entire length.

Experiment 4: In the initial experiments, 9 female pigs (n=12 tubed flaps) weighing 22 to 50 kg were used for development of the stage 1 procedure. It was found that all stage 1 tubed flaps survived to their entire length for periods ranging from 5 to 29 days. However, some degree of epidermal necrosis, followed by secondary intentional healing occurred if excessive subcutaneous tissue was included within the tubed flap; therefore, female pigs weighing in excess of approximately 35 kg are not suitable for preparation of single-pedicle, axial-pattern tubed flaps as described above. Although six tubed flaps in this series were prepared on three female pigs, it was found that delay between procedures (approximately 60 days) to allow, ostensibly, intussusceptive growth and release of skin tension at the first donor site, thereby allowing primary closure of the second donor site, was complicated by weight gain in the female pigs, which made tubed flap closure without tension difficult, even with maximal subcutaneous tissue debulking.

As of November 1985, 67 stage 1 tubed flaps have been successfully prepared and the operative technique described above has been adopted as a standard operating procedure for the preparation of IPPSF. Currrently, arterial cannulas (stage 2 procedures) have been prepared successfully in 69 of 71 axial-pattern skin flaps for either microangiographic studies (n=31), see Results, experiments 3 and 5), or isolated organ perfusion studies (n=38). In those two female pigs in which the stage 2 procedure was performed unsuccessfully, the CSEa was judged too small to cannulate. The principles of the operative technique as described above have been adopted as a standard operating procedure for the preparation of IPPSF. The venae comitantes have been successfully cannulated in two IPPSF using a commercially available microcatheter.

Based on measurements of tubed skin flaps at time 0, 2 days, and 6 days after stage 1 procedure, estimations of tubed flap skin surface area retraction/expansion can be made (Table 2).

Isolated Perfusion

Skin flaps harvested 2 or 6 days after stage I surgery remained viable for periods of 10 to 16 hours as assessed by glucose utilization, lactate production, absence of significant LD concentrations in the perfusate, a stable perfusate flow rate, and by LM and TEM. In two flaps perfused immediately after stage I surgery, vascular leakage occurred and criteria of viability were significantly poorer than in other flaps. Bacterial overgrowth occurred in two flaps, resulting in rapidly declining arterial pH, increased glucose utilization and the inability to maintain normal arterial oxygen tension. These indicators were succesfully used to predict overgrowth in later flaps prior to confirmation by standard clinical microbiological procedures.

Table 2: Measurements of stage 1 tubed flaps for determination of tubed flap skin surface retraction/expansion.

Parameter	Postoperative Time (days) 0†	2‡	6§
Length*	7.2 ± 0.1	7.4 ± 0.1	7.5 ± 0.2
Diameter*:			
Base:	1.4 ± 0.03	1.7 ± 0.03	1.6 ± 0.09
Tip:	1.4 ± 0.03	1.8 ± 0.05	1.7 ± 0.09
Calculated area‖:	31.7 cm^2	40.7 cm^2	38.9 cm^2
Percentage of original area¶:	...	128%	123%

* Dimensions in cm (* ± SE).
† n = 39
‡ n = 27
§ n = 6
‖ Based on formula: $\frac{(D_{base} + D_{tip})}{2} \pi \times \text{length} = \text{calculated area in } cm^2$
¶ Percentage of original, <u>in situ</u> dimensions (30.0 cm^2)

Bacterial isolates included Klebsiella oxytoca and Aeromonas hydrophila.

Data from 18 IPPSF's, which were harvested either 2 days (n=12) or 6 days (n=6) following stage I surgery, are summarized in Table 3. No major differences between 2 day and 6 day flaps were seen for any of the viability parameters listed in Table 3. Although statistically significant differences in terminal versus initial osmolality were found for both types of IPPSF ($p < 0.01$), the increases were small (<10%) and were probably a result of lactate accumulation in the perfusate. Both 2 day and 6 day flaps showed a significant weight gain over the course of a 10-12 hour study ($p < 0.01$), with 2 day IPPSF weight increasing approximately 25% and 6 day IPPSF weight increasing almost 42%. The exact nature of these increases is unknown; however, morphological evidence of mild dermal edema and hypertrophic growth was seen in most IPPSF's.

During the first 10-12 hours in all 18 preparations, the cumulative glucose consumption was linear, with squared correlation coefficients (R^2) between 0.97 and 0.999, indicating uniform glucose consumption with time (Figure Three). Further evidence of the viability of the IPPSF can be appreciated when glucose extraction is plotted against perfusate flow (Figure Four). In order for the IPPSF to maintain uniform glucose consumption over time, glucose extraction increased in direct proportion to decreased flow. In four earlier flaps in which constant arterial glucose concentrations greater than 80 mg/dl were not maintained by glucose infusion, glucose extraction was linearly correlated with arterial glucose concentration.

Flap death, first evident by 16 hours of perfusion in experiments not deliberately terminated at 10-12 hours (IPPSF's 1-3), was marked by a drop of glucose utilization and a plateau in the cumulative glucose consumption curve, concurrent with a rise in LD values. Terminal LD values during a viable period were relatively insignifcant at 10.3 $\pm$ 2.4 iu/L (range = 0 to 30 iu/L, while 8- to 100-fold increases usually coincided with flap death. In four flaps, sodium fluoride (10 mg/ml) was administered after 5 (n=2) or 11 (n=2) hours of perfusion. Approximately 40 to 90 minutes later, glucose utilization abruptly ceased, evidenced by a plateau in cumulative glucose consumption at the termination of these experiments (Figure Three).

Arterial lactate concentrations increased progressively throughout each IPPSF experiment and had linear correlations greater than 0.97 with the cumulative glucose utilization (Figure Five). Based on the regression of mmoles lactate produced versus mmoles of glucose consumed, the mean lactate:glucose ratio was 1.6 $\pm$ 0.2 for 2 day preparations (n=12) and 1.8 $\pm$ 0.2 for 6 day preparations (n=6). When arterial pH was not regulated by sodium bicarbonate infusions (five flaps), pH decreased over the course of a 12 hour experiment.

Table 3: Estimates of biochemical function in perfused skin flaps harvested either 2 or 6 days after stage I surgery.

Variable	Days After Stage I Surgery 2 (N=12)	6 (N=6)
Arterial Glucose Conc. (mg/dl)[a]	114.6 ± 4.6[b]	96.3 ± 5.6
Glucose Utilization (mg/hr)[a]	25.1 ± 2.7	26.2 ± 5.4
Flow (ml/min)[a]	1.9 ± 0.2	1.7 ± 0.4
Cumulative Glucose Consumption (mg)[c]	241.0 ± 24.2	199.9 ± 18.6
Terminal Lactate Conc. (mM)[c]	2.2 ± 0.1	2.5 ± 0.3
Terminal LD (IU/L)[c]	7.6 ± 2.5	16.9 ± 4.6
Initial Osmolality (mOsm/kg)	347 ± 18	331 ± 23
Terminal Osmolality (mOsm/kg)[c]	374 ± 24	387 ± 17
Initial Weight (g)	30.5 ± 1.1	30.3 ± 1.2
Terminal Weight (g)[c]	38.3 ± 1.8	42.9 ± 7.5

[a]Individual values represent the average of 8-12 observations per IPPSF.

[b]Mean ± SE.

[c]After 10-11 hours of perfusion.

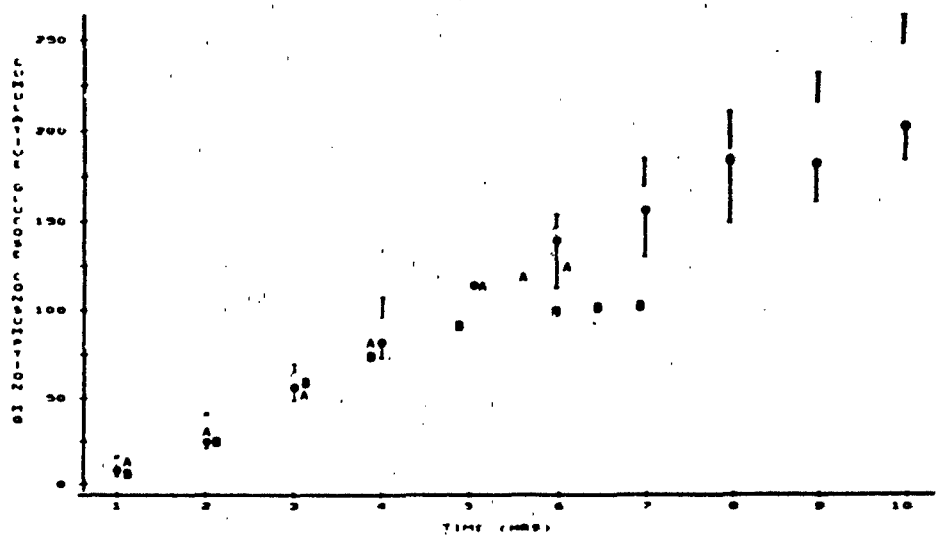

Fig. 3. Plot of mean cumulative glucose consumption versus time for flaps harvested 2 days (open circles, n=12) and 6 days (closed circles, n=6) after stage I surgery. The bars represent one SE. Two preparations receiving NaF at five hours are plotted individually (A,B).

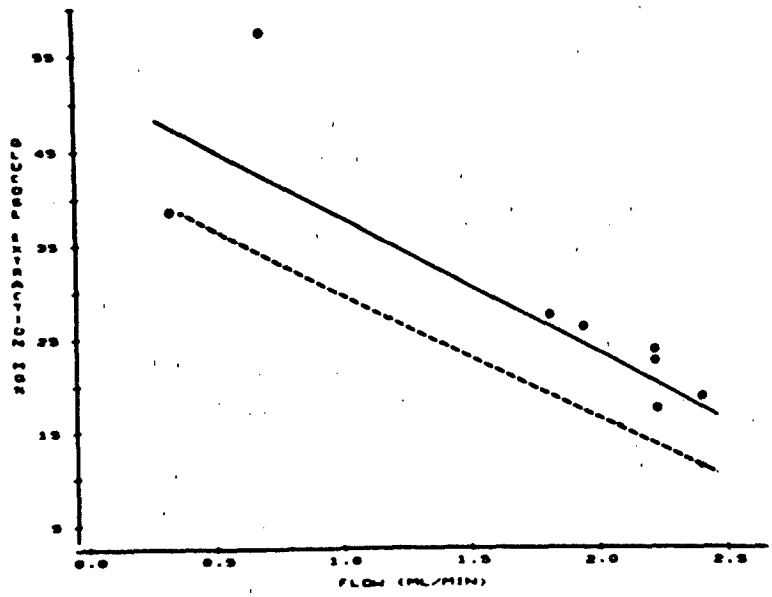

Fig. 4. Plot of glucose extraction versus perfusate flow rate for an IPPSF harvested 2 days after stage I surgery (open circles) and one 6 days after stage I surgery (closed circles).

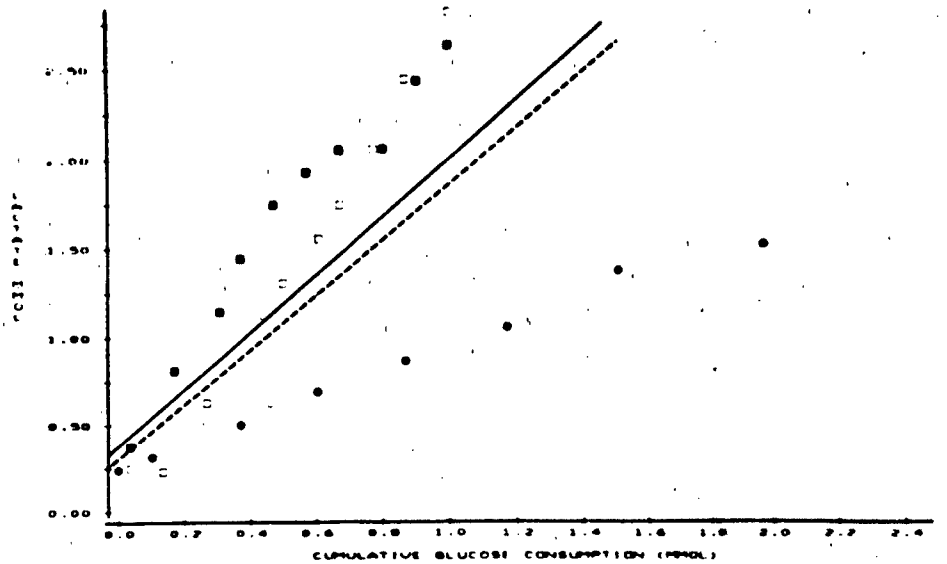

Fig. 5. Plot of glucose consumption versus lactate production for 4 IPPSF's which form an envelope for the remaining 14 IPPSF's. The flap represented by closed squares had the maximum slope and the one represented by closed circles had the minimum slope of all flaps harvested 6 days after stage I surgery. The solid line has slope and intercept equal to the mean slope and intercept for 6 days IPPSF's. The 2 day harvests' maximum (open squares), minimum (open circles), and regression line (broken line) are similarly defined.

Morphologic Studies:

LM evaluation on H&E sections collected from dying skin revealed noticeable changes occurring over time. A discriminant analysis revealed that the scores based on the counts of pyknotic basal cells, basal vacuoles, and pyknotic stratum spinosum contained information sufficient to correctly classify cell death. Including all six scores resulted in the correct classification of only two additional slides. Hence the sum of these three scores was used as an appropriate morphologic viability index, a decrease in viability being reflected as an increase in the index. The decline in viability was significantly different ($p < 0.05$) between the first three and the last two pigs. The best line fit through the data from pigs 1-3 had a slope of 0.45 as compared with a slope of 0.40 from pigs 4 and 5 (data not plotted). This difference, although small, is supported by examination of the change in subjective rating across time for the two groups. The shift from 1 to 2 (normal to transition) occurred between 2 and 3 hours for group 1 and between 5 and 12 hours for group 2. Similarly, the shift from 2 to 3 (transition to necrotic) occurred between 12 and 24 hours in group 1 and between 18 and 24 hours for group 2. Obvious abnormal LM changes occurred at 12 hours consisting of dark basal nuclei, pyknotic basal cells, basal vacuoles, dark stratum spinosum cells, pyknotic stratum spinosum cells, and stratum spinosum vacuoles. By 24 hours, some pyknosis and degenerative changes occurred within the epidermal cell layers. At 48 hours, the dermis was abnormal, being granular in appearance, and almost all the epidermal cells were pyknotic and epidermal-dermal separation occurred. TEM observations showed normal morphologic integrity until 8 hours. At 8 hours, morphological alterations consisted of large single vacuoles, disruption of mitochondrial membranes, chromatin-clumping with nucleolar segregation, and nucleolar pleomorphism occurring within the stratum basale and stratum spinosum layers. At 12 hours (Figure Six), nuclear envelope separation, single vacuoles, separation of desmosomes and degenerative organelles and debris were in the intercellular space, yet the stratum corneum remained intact. By 24 hours, classical pyknosis occurred with shrinkage of the nucleus and condensation of chromatin. Other changes associated with necrosis such as karyorrhexis, larger vacuoles, and at times lipid droplets near the mitochondrion were also observed.

In the <u>in situ</u> study, LM at day 0 after surgery demonstrated that the epidermis and dermis were normal. At day 1, edema was present in the superficial and deep dermis and chromatin-clumping was seen. Day 2, edema was present in both superficial and papillary layers of the dermis and lymphatic vessels were dilated. Slight intracellular edema and chromatin-clumping were present in the epidermal cell layers. Perivascular to diffuse eosinophilic mononuclear infiltrate occurred in the dermis. On day 3, similar changes were noted but dark stratum

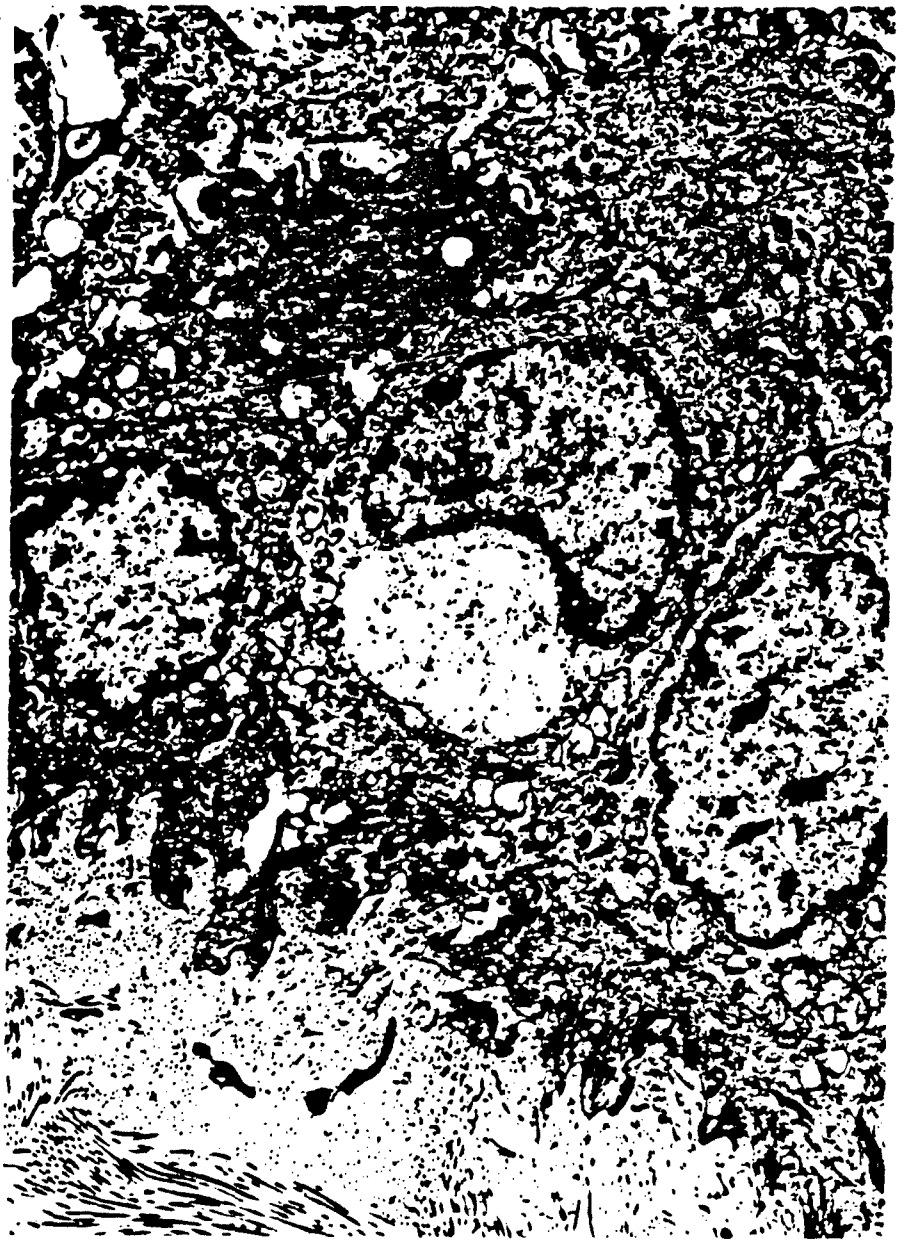

Fig. 6. Electron micrograph of detached skin at 12 Hours. Note nuclear envelope separation and single vacuoles. X8,800.

basale cells were also seen. Days 4, 5 and 6 were similar. By day 7, very slight intracellular edema of the epidermal cells was seen, perivascular and diffuse eosinophilic mononuclear infiltrates were present, but the superficial and deep dermis appeared normal.

From the morphometric analyses (Table 4), it is clear that the major difference attributable to the time between stage I and stage II surgeries is a thickened epidermal layer. By 7 days after stage I surgery, epidermal thickness had approximately doubled in both thin areas and at rete pegs, in addition to having greater variability over time, suggesting that the 2 day preparation may be more appropriate for percutaneous absorption studies. These data are also consistent with the hypertrophy seen in most 6 day IPPSF's. Ultrastructural observations at day 0 and day 1 were normal. On day 2, redistribution of nucleolar components into fibrillar and granular ribonucleoprotein filaments occurred. Several cells in mitosis were present in the stratum basale and spinosum layers. Some cells possessed a reticular nucleolonema with light granular and dense filamentous components and rounded pars amorpha areas or fibrous centers (Figure Seven). Day 3, the ultrastructure of the nucleolus resembled that on day 2 but tended to have an increase in the dense filamentous component. Day 4, irregularities developed within the nucleolus - that is, the irregular shape, a compact appearance and nucleolar margination. Day 5, the nucleolus was enlarged and consisted of dense filamentous components. Day 6, nucleolar margination was prominent and nucleolus was very much enlarged. Several cells of the stratum basale and spinosum were characterized by a compact appearance without a developed nucleolonema and uniform distribution of structures containing RNA. Other nucleoli of some cells consisted of a dense fibrillar center with a periphery of granular components. Day 7, the nucleoli of both the stratum basale and stratum spinosum cells resembled that of day 6. No nucleolar caps, microsegregation or macrosegregation was noted.

LM of the epidermis in IPPSF's terminated after 12 hours of perfusion demonstrated normal intact epidermis (Figure Eight). To assess individual cellular morphology, TEM was also employed. Nucleolar pleomorphism was present in the stratum basale and spinosum layers (Figure Nine). Some vacuolization was also observed, depending on the location of the sample on the IPPSF. However, degenerative changes such as those associated with cell death in the control study were not seen.

Finally, TEM on IPPSF's receiving NaF revealed normal epidermis except for the presence of large multiple vacuoles, often membrane-bound, and at times showed an amorphous substance in the stratum basale and spinosum layers (Figure Ten). These changes seen in in the NaF flaps should be considered acute toxic lesions rather than degenerative changes secondary to loss of viability.

Table 4: Morphometric analyses of epidermal thickness[a] in *in situ* skin flaps (n=3) at various times between stage I and stage II surgeries

Day	Epidermis (um)	Rete Pegs (um)
0	37.1 ± 2.8[b]	65.8 ± 16.9
1	43.5 ± 2.8	80.0 ± 13.0
2	68.1 ± 6.8	102.3 ± 4.4
3	82.9 ± 15.6	141.3 ± 12.0
4	67.4 ± 12.7	178.4 ± 13.8
5	96.4 ± 7.2	165.4 ± 11.8
6	86.2 ± 16.0	169.1 ± 23.5
7	68.1 ± 11.0	152.9 ± 38.3

[a]See Materials and Methods for a description of techniques used. Stratum corneum thickness did not vary and is not included in epidermal measurements reported here.

[b]Mean ± SE in three *in situ* flaps.

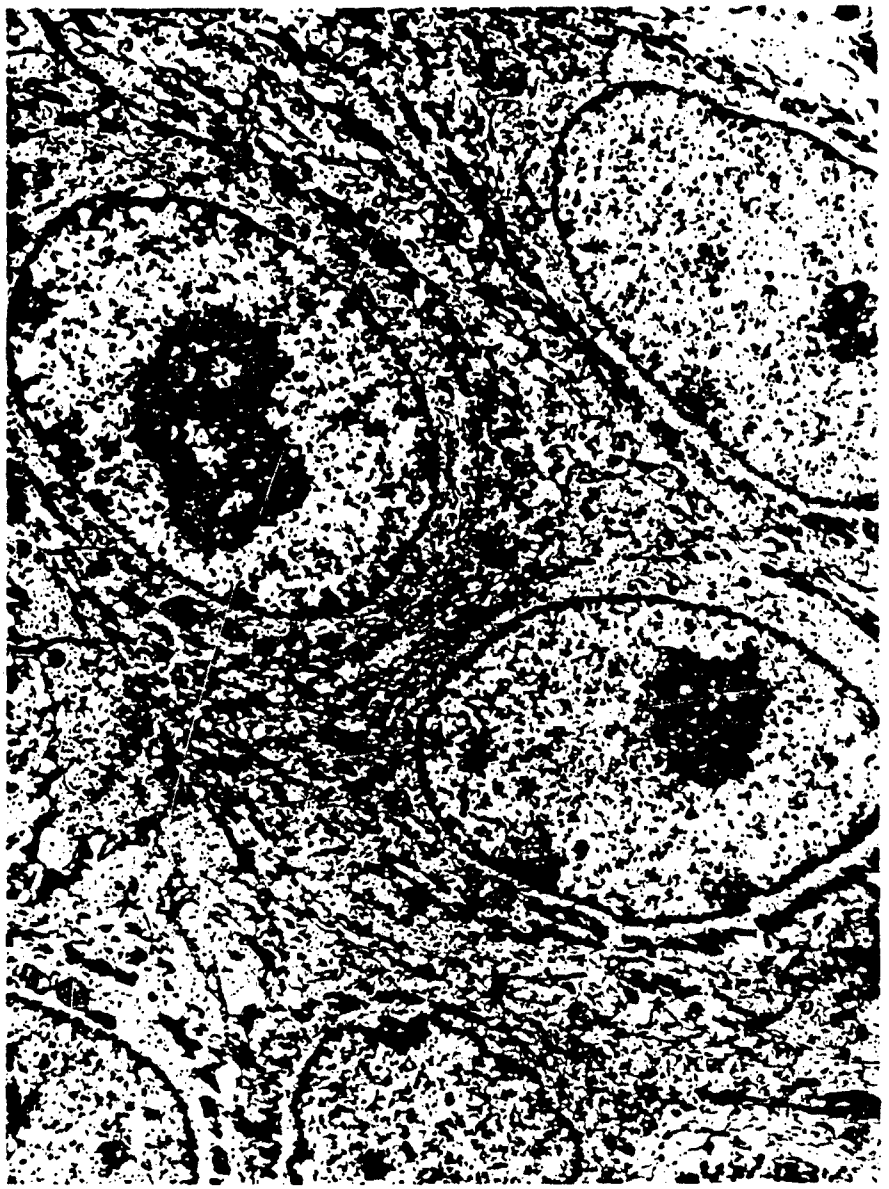

Fig. 7. Electron micrograph of a two day *in situ* flap after surgery. Note the nuclear redistribution. X8,000.

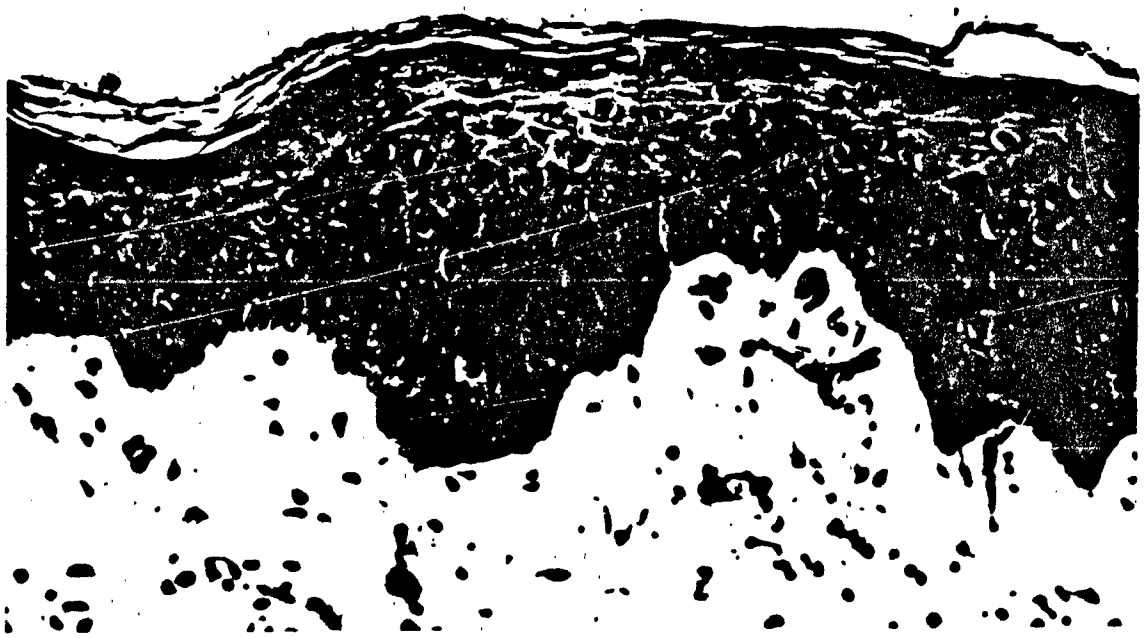

Fig. 8. Light micrograph showing viable epidermis and dermis in an IPPSF harvested 2 days after stage II surgery. H&E, X100.

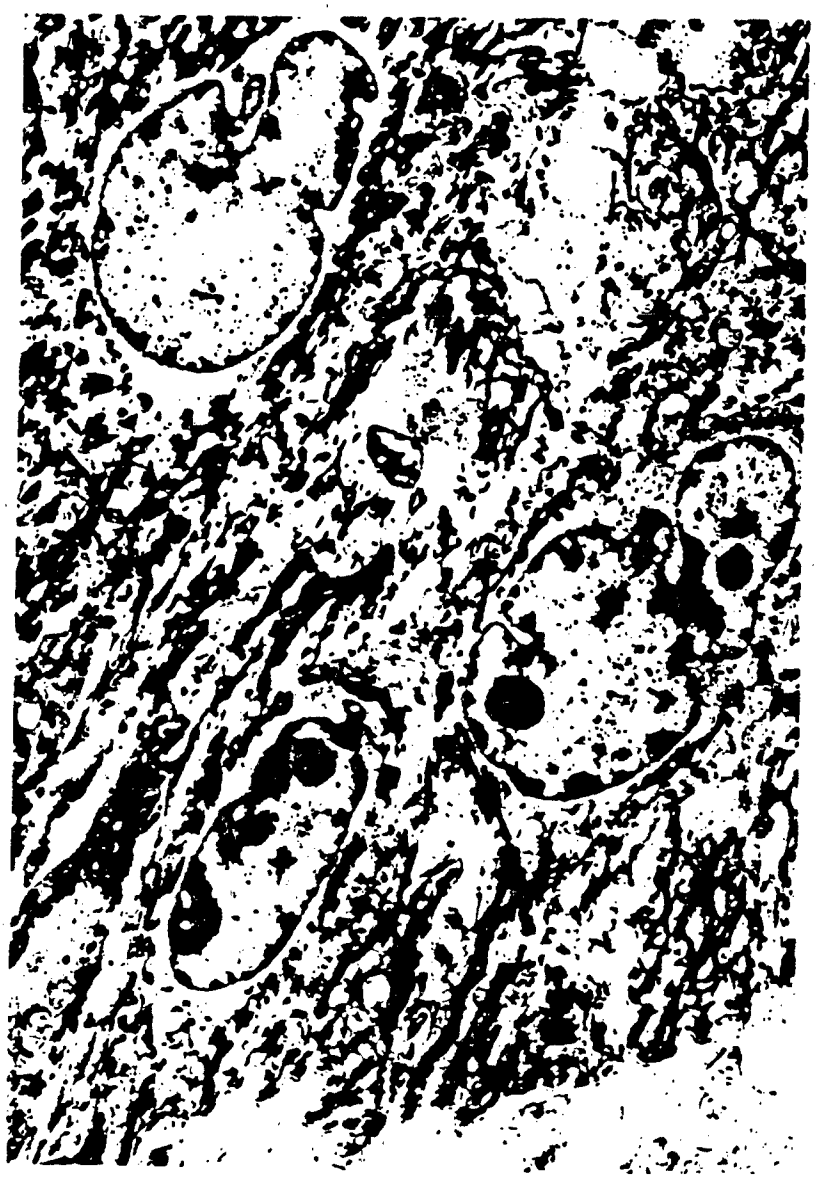

Fig. 9. Transmission electron micrograph of a viable IPPSF after 12 hours of isolated perfusion. Note the nucleolar pleomorphism in the stratum basale layer. X5,400.

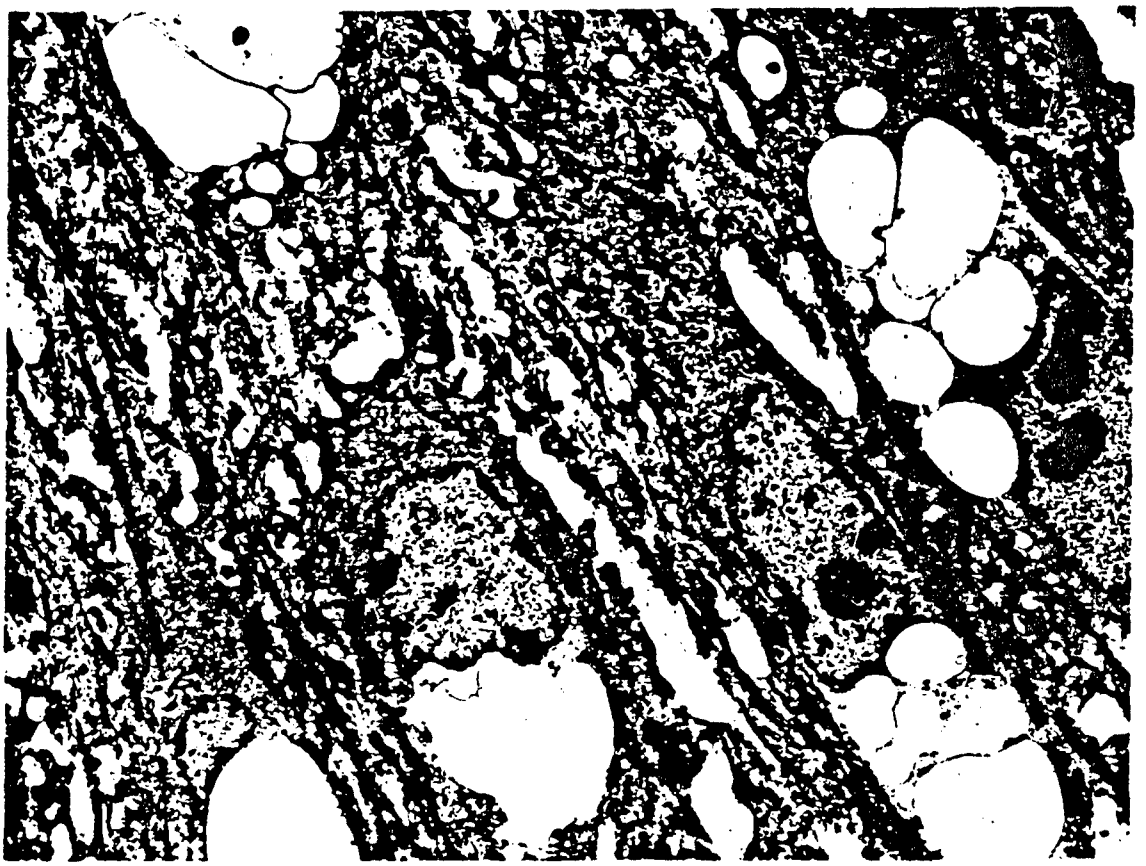

Fig. 10. Electron micrograph of an IPPSF administered sodium fluoride. Note the multiple vacuoles. X4,800.

DISCUSSION

These experiments demonstrate the feasibility of maintaining a viable skin preparation for 10-12 hours in an isolated organ system. The finding of increasing lactate concentrations linearly correlated to glucose utilization over the course of an experiment is consistent with other in vitro studies in skin demonstrating that anaerobic glycolysis is a primary bioenergetic pathway for skin and that lactate is the primary end-product of epidermal glycolysis (25). In this preparation, an overall mean lactate/glucose ratio of 1.7 suggests primarily anaerobic glycolysis and a smaller component due to non-lactate-producing pathways. The linear glucose consumption over time maintained by an extraction ratio inversely proportional to perfusate flow indicates a stable, self-regulating metabolic system. Consistent with previous skin studies (26), glucose extraction was directly proportional to available glucose concentrations in the four flaps in which constant glucose concentrations were not maintained above 80 mg/dl. Sodium fluoride inhibition of glucose utilization, coupled with morphological indications of an anatomically normal epidermis, is further evidence of the viability of this preparation.

Morphologic assessments are a valuable tool to differentiate lesions due to various factors in the IPPSF. The results from the LM analysis to assess viability appear to be insufficient for the development of a morphologic index because of animal and site-site variability. In contrast, morphologic criteria using TEM are essential for the differentiation of changes due to cell death, postoperative procedures, isolated perfusion,and xenobiotically induced toxicity.

The continued accumulation of lactate and other waste products in this closed system probably contributed to decreased function with time in flaps not terminated at 10-12 hours. However, 10 hours of viability is considerably longer than the 4-6 hours generally seen in isolated liver and kidney preparations (27). Perfusate exchange, addition of a membrane dialyzer into the system, or forced utilization of lactate as an energy source by hormonal stimulation may significantly extend the viable period of this preparation. Based on basic biochemical principles (28), it is our hypothesis that incorporation of amino acids or short-chained fatty acids into the perfusate may also prolong viability. At present, a period of 10-12 hours should be adequate for modeling the initial rapid phase in percutaneous absorption studies, the phase which is critical in cases of acute intoxication. Finally, based on the biochemical data, there is no difference between IPPSF's harvested 2 or 6 days after stage I surgery, except for an increased tendency to gain weight during the experiment in 6 day IPPSF's. Morphologically, by 6 days the epidermis appears to be much thicker than at 2 days and the

interflap variability is also greater. Because the IPPSF is intended for percutaneous absorption studies, 2 day flaps appear to be optimal.

As presently configured, the IPPSF may be useful for a number of investigations. The ability to place drugs or chemicals on the surface of viable skin maintained in a controlled environment, combined with the measurement of arterial and venous drug concentrations, makes this preparation well suited for investigating the percutaneous absorption of drugs and chemicals. If first-pass cutaneous metabolism of the compound occurs, the IPPSF should allow for the identification of metabolites produced by epidermal processes and be useful for quantitating the rate of these processes for incorporation into recently proposed pharmacokinetic models (29,30,31). Present in vivo studies are confounded by the biotransformation of absorbed agent in the liver and kidney, while in vitro techniques using cadaver skin are not applicable because of its limited viability (1,32). The IPPSF would also allow quantitation of the effects of temperature, altered cutaneous microcirculation, disease, bioenergetic profile and decontamination strategies on percutaneous absorption. The IPPSF preparation appears well suited for studying the normal biochemistry of the skin. If IPPSF's were raised in swine breeds which possess spontaneous cutaneous melanomas (33), an isolated perfused vascular bed containing normal and cancerous tissue would be available for various studies in cancer biology. Finally, the IPPSF is an alternative animal model which should allow cutaneous toxicology studies to be conducted in a humane manner.

The IPPSF would have a number of uses relevant to military needs. The pharmacokinetics and cutaneous biotransformation of topically exposed CW agent could be studied independent of systemic toxic effects. Decontamination protocols for cutaneous agent exposure could be assessed. The design of transdermal drug delivery systems for CW nerve agent prophylaxis would be greatly facilitated since the data collected in the IPPSF would be appropriate for direct incorporation into existing pharmacokinetic models. Finally, the IPPSF might be ideally suited for studying the mechanisms of cutaneous toxicity induced by the vessicant CW agents mustard and lewisite, since early biochemical and morphological changes could be assessed in an isolated viable skin preparation with a responsive microcirculation. Decontamination and protection strategies against vessicant agents could then be humanely evaluated in the IPPSF.

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